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Published in:
Journal of Neuroscience Methods

DOI:
[10.1016/j.jneumeth.2019.108323](https://doi.org/10.1016/j.jneumeth.2019.108323)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Peleh, T., Bai, X., Kas, M. J. H., & Hengerer, B. (2019). RFID-supported video tracking for automated analysis of social behaviour in groups of mice. *Journal of Neuroscience Methods*, 325, [108323]. <https://doi.org/10.1016/j.jneumeth.2019.108323>

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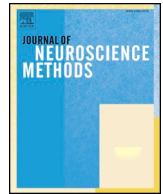
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RFID-supported video tracking for automated analysis of social behaviour in groups of mice

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ARTICLE INFO

Keywords:

Automated
Mouse tracking
Social behaviour
Video tracking
RFID

ABSTRACT

Background: Deficits in social behaviour, e.g. social withdrawal, appear as an early sign of many neuropsychiatric disorders. Investigation of the biological basis of social withdrawal and development of new targets for treatment requires reliable quantification methods of social behaviour.

New method: In order to study behavioural deficits in preclinical rodent models, we developed a tracking and analysis tool for behavioural observations in groups of mice. *RFID-Assisted SocialScan* is based on video tracking supported by radio-frequency identification (RFID). For this purpose, mice were labelled with RFID tags providing unique animal identity and location in the arena. An integrated software package enables automatic detection of predefined behavioural events, which are extracted from video recordings. We designed a social arena that can be flexibly adapted for various behavioural experiments.

Results: We demonstrate the utility of our newly developed tracking tool by monitoring colonies of C57BL/6 J mice. We assessed social (approach, contact, follow, leave) and locomotor activities over multiple days.

Comparison with other existing methods: *RFID-Assisted SocialScan* is an automated tracking and analysis tool for long-term behavioural observations of multiple freely moving mice housed in ethologically relevant environment.

Conclusions: Here, we demonstrate the performance of a newly developed behavioural tracking system that can be used for long-term translational studies of social behaviour in groups of freely moving mice.

1. Introduction

Social behaviour is involved in many aspects of life, e. g. communication, social play (social learning), reproduction and parental behaviour. Deficits in social behaviour such as social isolation and social withdrawal can be first signs of many neuropsychiatric disorders (for a review, see Porcelli et al., 2019). In preclinical research, social deficits in mice are mainly assessed in short-term assays without direct physical contact. For instance, in the three-chamber social approach task the test subject is given the choice to approach a locally restricted conspecific or an empty container (Crawley, 2007; Moy et al., 2004; Nadler et al., 2004). However, this type of behavioural assay represents rather a snapshot of one aspect of social behaviour in mice. To capture a broader repertoire of social behaviours and to investigate its dynamics over long time periods, monitoring of freely moving mice under more natural or home-cage-like conditions is required. However, assessing social

interactions in freely moving mice is challenging. Though, manual scoring is still a widely used method of behavioural analysis, it is labour intensive, time consuming and limited to human eye performance. For these reasons, tracking solutions for automated behavioural quantification have been developed (for a review, see Peleh et al., 2019). Most tracking systems are based on video recording and automatically deliver information about animal's location, distance travelled and velocity (e.g., Aguiar et al., 2007; Crispim Junior et al., 2012; de Visser et al., 2006; Spink et al., 2001; Tort et al., 2006). A common problem in video tracking is the inability to maintain the identity of multiple animals. Whenever two subjects are in close proximity, their identities may swap. Some video tracking systems allow for discrimination between two animals based on constantly visible labelling. However, visible colour labelling suffers from tracking loss when colour label is invisible to the camera, is difficult to realize under infrared light (e.g. when tracking in the dark) and may influence the behaviour of the animals.

Abbreviations: RFID, radio-frequency identification; Sc, subcutaneously

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<https://doi.org/10.1016/j.jneumeth.2019.108323>

Received 23 April 2019; Received in revised form 11 June 2019; Accepted 24 June 2019

Available online 27 June 2019

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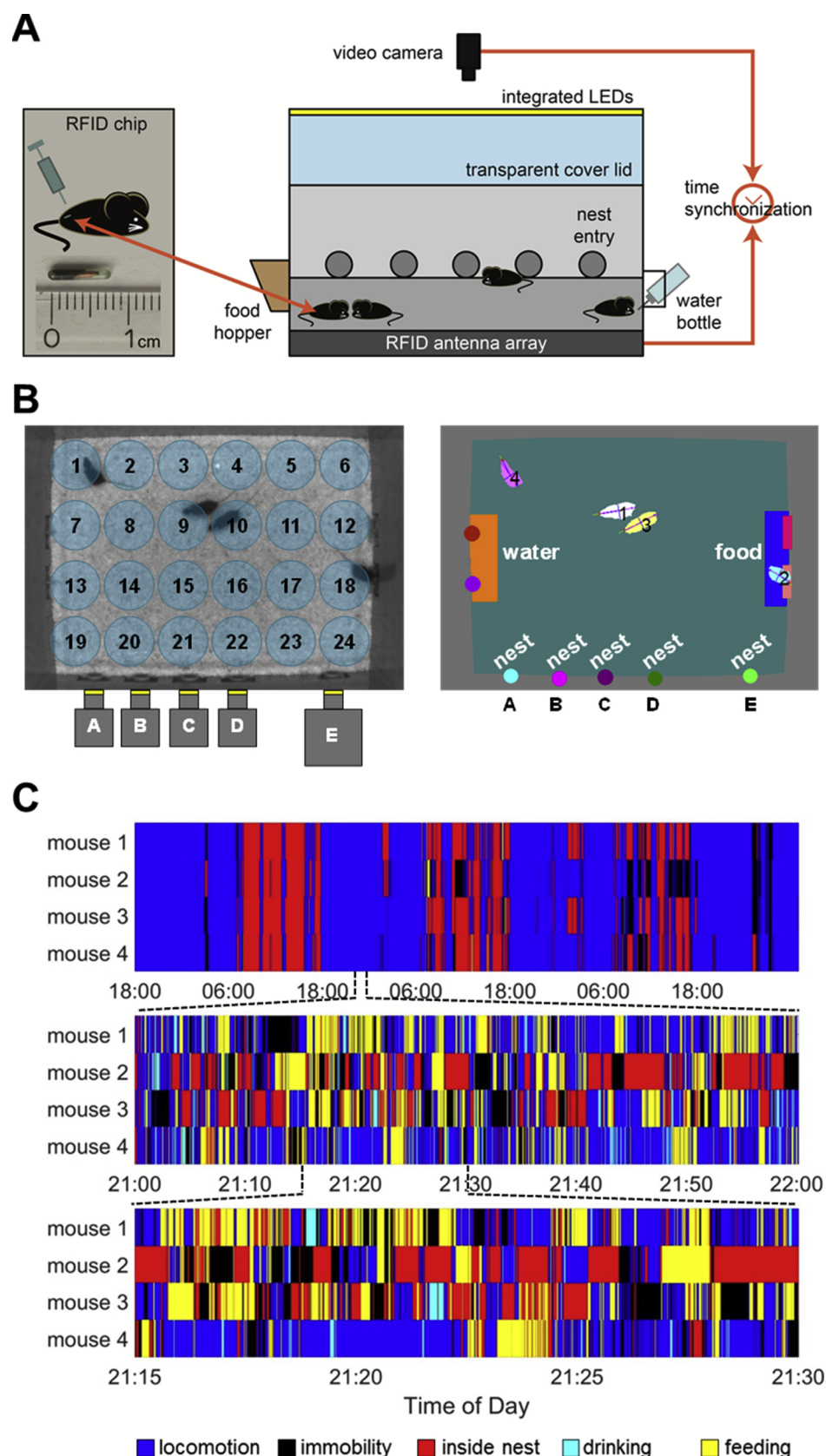


Fig. 1. Automated behaviour detection of individual mice using video tracking supported by RFID. (A) Schematic representation of *RFID-assisted SocialScan*. The system is composed of a video tracking tool (camera on top) supported by an array of RFID antennas underneath the arena. Video frames are synchronized with RFID signals and individual mice are automatically identified by a subcutaneously implanted RFID chip (1.25 mm × 8 mm). (B) *Left*: Top view of the social arena (60 cm × 80 cm) equipped with five nests (A–E), two water bottles and two food hoppers. Blue circles represent 24 RFID antennas located underneath the arena floor (= RFID antenna array). Additional RFID antennas are connected at each nest entry (yellow) to detect animals outside the camera view. *Right*: Illustration of user defined detection zones marking arena floor, nest entries, water zone and food zone. Mouse IDs (1–4) are automatically assigned based on unique numbers of the RFID chips. (C) Time line representing a small section of automatically detected behaviours in individual mice over the course of 84 h (*top*), 1 h (*middle*) and 15 min (*bottom*). Using *RFID-Assisted SocialScan*, data can be automatically represented at different temporal granularities showing various degrees of detail such as sequence of particular behavioural events.

Other tracking systems overcome obstacles of visual discrimination by using radio-frequency identification (RFID) (e.g., [Catarinucci et al., 2014](#); [Endo et al., 2011](#); [Howerton et al., 2012](#); [Lewejohann et al., 2009](#); [Macri et al., 2015](#); [Puścian et al., 2016](#)). Rodent RFID tracking

tools use passive RFID operating at low frequencies (125–134 kHz). Mice are injected subcutaneously (sc) with a small RFID transponder detected by RFID antennas in close proximity (within a reading scope of a few centimetres). Passive RFID has a low spatial resolution and gives

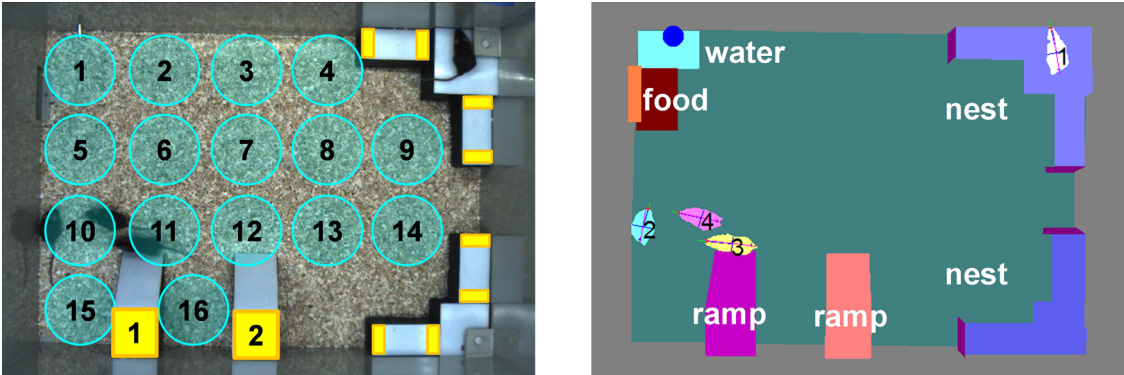


Fig. 2. Automatic tracking of individual mice in an arena with complex environment. *Left:* Behavioural arena (50 cm × 70 cm) equipped with 16 RFID antennas under the arena floor, two nests with RFID antennas (2 per entrance) and two ramps with antennas. Mice were injected sc with RFID chips (1.41 mm × 9 mm). *Right:* Illustration of detection zones including nests, ramps, water and food area.

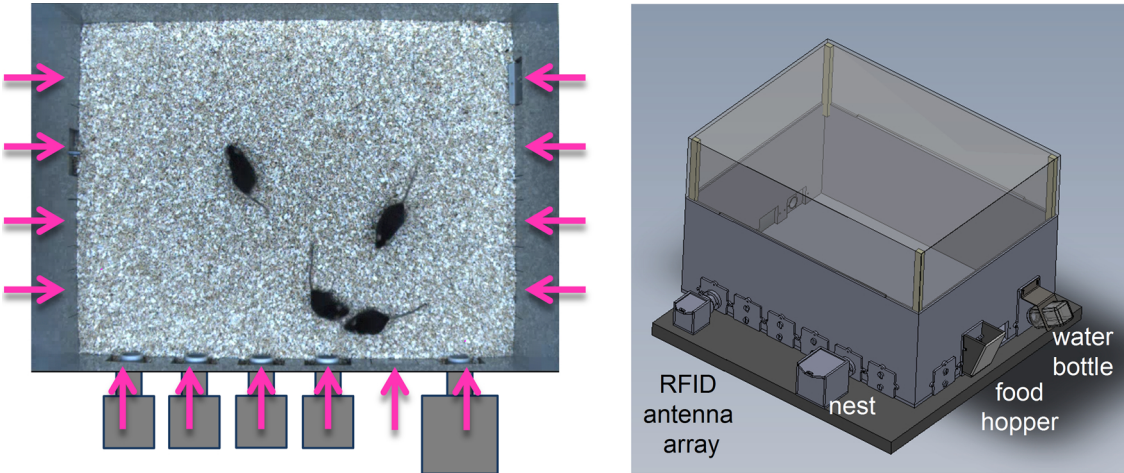


Fig. 3. Flexible behavioural apparatus with modifiable degree of complexity. *Left:* Top view of the social arena (60 cm × 80 cm) with 14 possible connections (pink arrows) to flexibly install nests, food hoppers, water bottles and other devices to increase complexity of the environment. *Right:* 3D drawing of the social arena equipped with nests, food and water supply.

Table 1

Behavioural parameters.

Behaviour	Definitions
<i>Social contact:</i>	<i>Mouse 1 and mouse 2 in close proximity:</i> Distance between mice < 1 cm Minimum Duration 0.5 sec
<i>Social approach:</i>	<i>Mouse 1 approaches mouse 2:</i> Distance between mice < 20 cm Moving direction (angle) of mouse 1 < 30 ° Distance to be travelled by mouse 1 towards mouse 2 > 10 cm Velocity of mouse 1 > 3 cm/s
<i>Social leave:</i>	<i>Mouse 1 moves away from mouse 2:</i> Distance between mice < 10 cm Moving direction (angle) of mouse 1 > 30 ° Distance to be travelled by mouse 1 away from mouse 2 > 10 cm Velocity of mouse 1 > 3 cm/s
<i>Social follow:</i>	<i>Mouse 1 follows mouse 2:</i> Distance between mice < 20 cm Moving direction (angle) of mouse 1 < 30 ° Moving direction (angle) of mouse 2 > 90 ° Distance to be travelled by mouse 1 and mouse 2 > 10 cm Velocity of mice > 4 cm/s
<i>Locomotion:</i>	Distance to be travelled: > 1 cm Velocity > 1 cm/s
<i>Immobility:</i>	Distance to be travelled: < 1 cm Velocity < 1 cm/s Wobbling factor: 0.1 (value between 0 and 1 describing motion in place)

little information about animal's orientation, body posture and vigorous movements. However, RFID systems can reliably detect large number of animals over long periods of time. Consequently, a tracking tool would mostly benefit from a combination of multiple tracking techniques, such as computer vision, depth sensors, RFID, and/or machine learning (de Chaumont et al., 2018; Hong et al., 2015; Perez-Escudero et al., 2014; Shemesh et al., 2013; Weissbrod et al., 2013, for a review, see Peleh et al., 2019).

Here, we describe a novel automated tracking and analysis tool for behavioural observations in groups of mice: *RFID-Assisted SocialScan* (Fig. 1). The system integrates video tracking with RFID and reliably identifies individuals among groups of four mice without the use of visible marking. The hardware is composed of a video camera placed on top and a RFID antenna array placed underneath the behavioural apparatus (Fig. 1A). Each mouse is tagged with a RFID chip that is uniquely identified by the RFID antenna array. The software package synchronizes video frames with RFID signals and automatically identifies numerous events and behaviours of individual mice over long period of time. Behavioural data can be represented and stored at different temporal granularities (hours, minutes, seconds) yielding various degrees of information, e.g., in which sequence particular behaviours occur (Fig. 1C). Behaviours and events can be defined by the user by adjusting given parameters relevant for detection (Fig. 4). *RFID-Assisted SocialScan* can be used with various types of behavioural apparatuses. Therefore, we have constructed a social arena that can be flexibly adapted to different experimental conditions (Fig. 3). To demonstrate

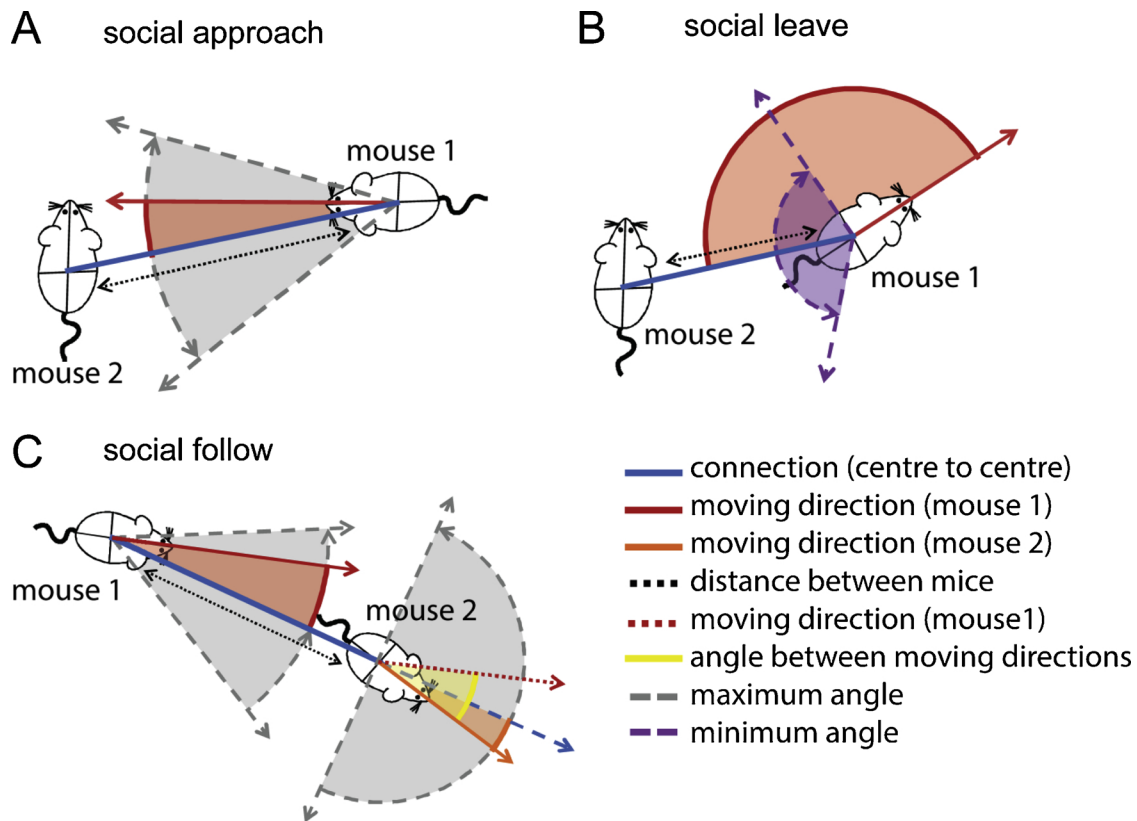


Fig. 4. Adjustable parameters for definition of social behaviours.

Social approach (A), social leave (B) and social follow (C) can be defined by a series of parameters considering moving direction, velocity, distance travelled and distance between mice over a sequence of frames.

the usability of our system, we monitored groups of male C57BL/6 J mice in a home-cage-like environment (Fig. 1B).

2. Material and methods

2.1. Animals

Experiments were conducted under the approval by the responsible governmental animal ethics committee (Regierungspräsidium Baden-Württemberg, Tübingen, Germany).

24 male C57BL/6 J mice (10 weeks old, Charles River Laboratories, Germany) were group-housed in IVC cages (Type 500) in a temperature ($22 \pm 2^\circ\text{C}$) and humidity ($\pm 45\text{--}65\%$) controlled room with 24 h light-dark cycle (12 h of lights on starting at 6:00 h, and 12 h of lights off starting at 18:00 h).

2.2. Behavioural apparatus and data collection

In the design phase of the project, a behavioural apparatus (Fig. 2) was constructed based on a publication by Shemesh et al. (2013). The aim was to identify individual mice in a complex environment using RFID-supported video tracking. The first arena consisted of a grey (polymer) apparatus ($50\text{ cm} \times 70\text{ cm} \times 30\text{ cm}$) and a transparent cover lid ($50\text{ cm} \times 70\text{ cm} \times 20\text{ cm}$). The arena was equipped with two integrated nests (big nest: $9\text{ cm} \times 9\text{ cm} \times 9\text{ cm}$; small nest: $7\text{ cm} \times 7\text{ cm} \times 7\text{ cm}$). Each nest was connected to two entrance areas ($10\text{ cm} \times 3\text{ cm} \times 4.5\text{ cm}$) at two sides of the nest. The entrance areas were equipped with RFID antennas at the front and at the rear, respectively. Two ramps (height: 10 cm) were installed with RFID antennas on top of the ramp platform ($6.5\text{ cm} \times 7\text{ cm}$). A food hopper and a water bottle were connected to provide animals with food and water ad libitum. Arena floor was covered with sawdust bedding (same as in home cage). The

RFID board placed underneath the arena was made of 16 circular RFID antennas organized in an array covering the floor of the arena. Nest and ramp antennas were connected to the main RFID board. Videos were recorded from above using light sensitive colour camera (Manta G-235C, Allied Vision, 30 frames per second, 720×480 pixels). Integrated LED lights provided a 12 h light-dark cycle with 21 lx during light and < 1 lx during dark phase. Mice were subcutaneously implanted with RFID chips ($1.41\text{ mm} \times 9\text{ mm}$).

Following the implementation of the first arena, a modifiable behavioural apparatus was constructed to flexibly change environmental conditions in the arena (Figs. 1B and 3). The motivation for the design of a novel behavioural arena was to increase the applicability of our system. The modifiable arena consisted of a grey (polymer) apparatus ($60\text{ cm} \times 80\text{ cm} \times 30\text{ cm}$) and a transparent cover lid ($60\text{ cm} \times 80\text{ cm} \times 20\text{ cm}$) (Fig. 3). The apparatus contained 14 small windows ($6\text{ cm} \times 8\text{ cm}$) to connect additional equipment such as nests (pink arrows in Fig. 3). For our experiment, we attached five houses in different sizes (one big and four small ones), two food hoppers and two water bottles to the apparatus (as shown in Fig. 1B). Spare windows were closed with specially designed plugs flush with the arena wall. Arena floor was covered with sawdust bedding (same as in home cage). An integrated and automatically controlled light source above the arena provided a 12 h light-dark cycle (21 lx during day and 0 lx during night phase). Additionally, IR lights (850 nm) were used for monitoring in the dark. Videos were recorded from the top using an IR camera (Basler, acA1300-60gmNIR, 30 frames per second, 720×480 pixels). The apparatus was placed on top of the RFID antenna array, composed of 24 strategically placed RFID antennas. Each RFID antenna coil was of 62 mm diameter with 140 mm between neighbour coils. Custom manufactured RFID array reader ensured reading distance greater than 60 mm (above the coil) for standard $1.24\text{ mm} \times 8\text{ mm}$ RFID tag, while working height over 40 mm is recommended. Additional RFID antennas (41–43 mm in diameter) were

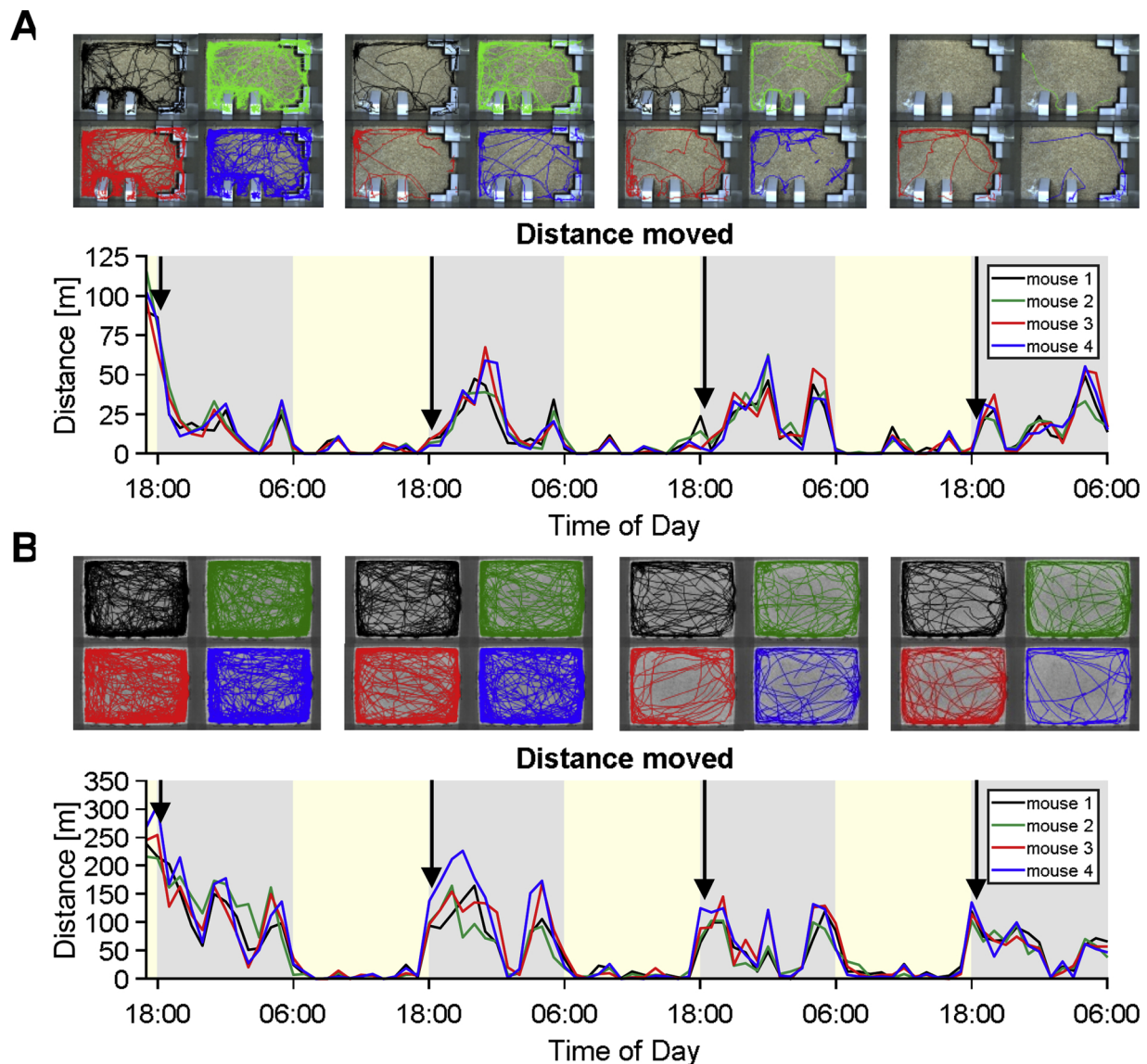


Fig. 5. Tracking of individual mice in different environments. Total distance (m) per hour travelled by two representative groups of four each male C57BL/6 J mice in two differently equipped social arenas. (A) Arena with integrated nests, ramps, food and water supply (for details see Methods Section 2.2 and Fig. 2). (B) Flexibly modifiable social arena with multiple nests attached from outside, water and food supply (for details see Method Section 2.2, Figs. 1B and 3). Tracking paths generated for each mouse display one hour at the beginning of dark phase (indicated by arrows), respectively.

placed at the entrance of each house (five in total) and connected to custom entry reader. All these readers, including array readers and entry readers, were connected to a central control unit which communicated with the computer. RFID readouts were picked up for each video frame, and integrated with video analysis to verify ID for each animal. The RFID system operated at 134.2 kHz. A distributed power system of 5 V DC with up to 10A was equipped to the RFID board.

2.3. Experimental procedure

One week prior to testing, mice were anesthetized with isoflurane (4%) and injected subcutaneously with RFID transponders (1.41 mm × 9 mm or 1.25 mm × 8 mm). RFID chips provide a steady signal when placed perpendicular to the antenna. To achieve a slightly tilted position of the chip, we injected at the lower back of the mouse (Fig. 1). Sterile and ISO compliant (ISO 11784/11785 FDX-B) RFID transponders were used only. We confirm that mice did not show any health or motoric issues after implantation of RFID tags (at least for 12 months after application). Groups of four unfamiliar mice were placed

in the arena one hour before the start of the dark phase. Video and RFID were recorded over 84 consecutive hours. Two colonies (n = 8) were used to demonstrate detection of individual mice in two differently equipped arenas. Four colonies (n = 16) were used for locomotion and social behaviour detection in a flexibly arranged arena.

2.4. Automatic analysis of behaviours

Behavioural analysis was conducted using a software package specially designed for use in our behavioural apparatus. The software (and the RFID antennas) is now commercially available under the trade name of *RFID-Assisted SocialScan* (CleverSys Inc., Reston, USA). The software package allows the user to create an analysis template including regions of interest (zones) and to select, define and modify behaviours for analysis. We created zones around water, food and nest entries (Figs. 1B and 2) and defined locomotion, immobility and social behaviours including contact, approach, follow and leave (Table 1). Behavioural events were represented in the software interface by a set of adjustable parameters such as moving direction angle, velocity,

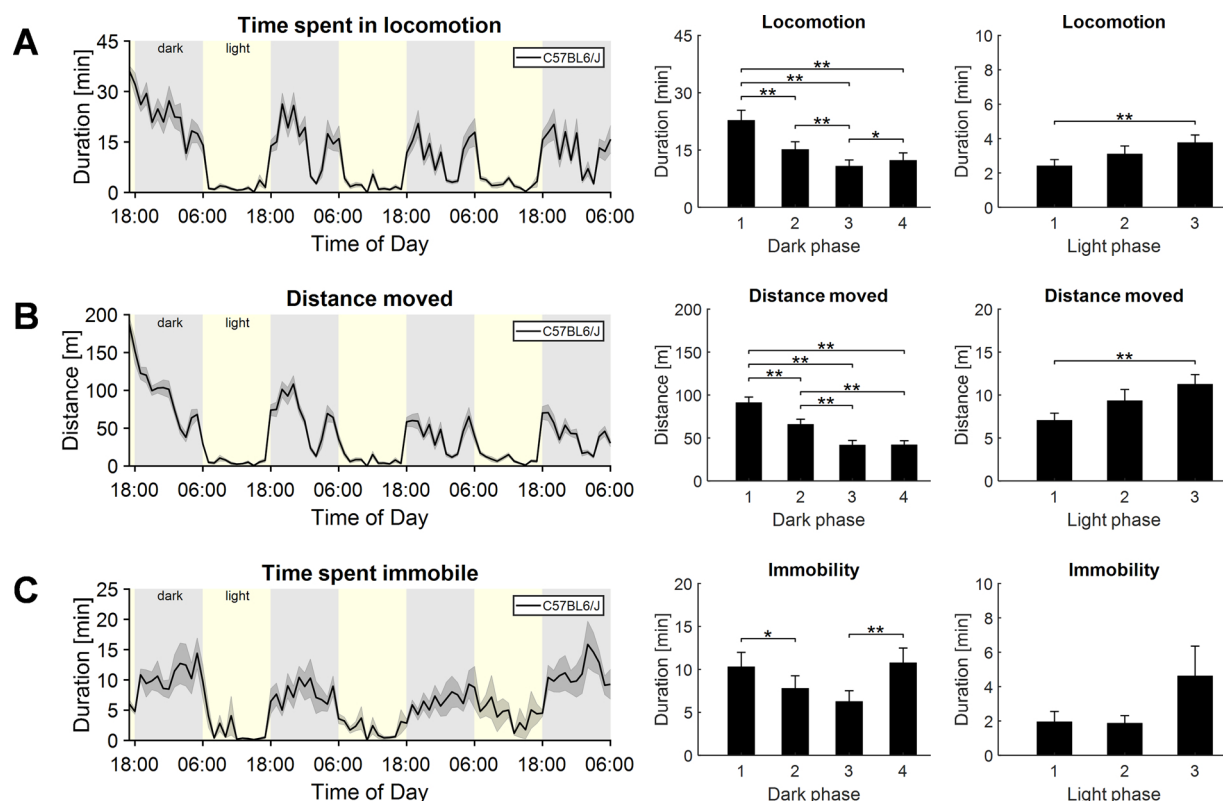


Fig. 6. Locomotion in groups of C57BL/6 J mice. Time spent in locomotion (A), distance moved (B) and time spent immobile (C) in groups of C57BL/6 J mice. Line graphs represent mean duration per 1 h over 84 h. Bar charts display mean duration per dark/light phase. Data are mean (\pm SEM) with $n = 16$ (with 4 mice per colony). * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA and Tukey's post hoc test.

distance between subjects and distance to be travelled. For instance, social approach is defined by the moving direction angle between the moving direction of the approaching subject (mouse 1, red solid arrow) and the centre point of the subject to be approached (mouse 2, blue connection line) (Fig. 4A). Thereby, the approaching subject (mouse 1) has to be within a certain radius of mouse 2 (distance between mice, black dotted line). Furthermore, mouse 1 has to travel a certain distance with a certain velocity towards mouse 2 (distance to be travelled by mouse 1) (Table 1). Social follow and leave are defined in a similar way (Fig. 4B and C). All behavioural definitions applied for our experiment are summarized in Table 1 and were manually annotated by the user. Videos were then uploaded together with the user defined analysis template and analysed in batch mode. The software extracted the animal's shape from video frames by background subtraction and detected multiple body points such as nose, body centre and tail of mice (Figs. 1B and 2). Mouse IDs were assigned automatically based on synchronously aligned video frames and RFID data, which contained time stamp, RFID transponder code (= mouse identity), and corresponding RFID antenna. When animals touched or crossed paths, identity swaps were subsequently and automatically corrected by the software. Data was exported in csv format, stored in a structured query language (SQL) database and processed using MATLAB (R2018a) and GraphPad Prism 8.0.

2.5. Statistical analysis

The data are expressed as mean (\pm S.E.M.) of time or distance moved in the arena. The data was analysed using Prism 8.0 (GraphPad Software, San Diego, CA, USA) and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for pairwise significances. The level of significance was set at $p < 0.05$ and indicated on the figures with single asterisks (*) or double asterisks (**) at $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Detection of individual mice in different environments

We measured the activity level in groups of male C57BL/6 J mice in two differently equipped arenas (Figs. 1B and 2). Fig. 5A shows locomotor activity of a representative C57BL/6 J group in a social arena designed on the basis of a previous publication (Shemesh et al., 2013). Fig. 5B displays a representative mouse colony in a newly designed social arena. In both arenas, mice showed rhythmic activity level with high locomotion during the dark phases and low locomotion during the light phases as expected from nocturnal animals. The longest distance was travelled by all four mice in the second hour (first hour of the dark phase) after introduction to the arena for the first time. In the first arena, mice explored the new environment by moving mainly along the walls and in close proximity to the ramps. In the second arena, mice moved homogeneously across the entire central area as it is shown by the tracking paths. During the first hour of the following dark phases, mice moved mainly goal-orientated between food, water and nest entries in both arenas.

3.2. Locomotion in a flexibly arranged arena

We measured the activity level in 4 colonies ($n = 16$) of male C57BL/6 J mice over 84 consecutive hours (Fig. 6). We observed a significant decrease in locomotor duration (one-way ANOVA, $F_{2,26} = 53$, $p < 0.001$) and distance moved (one-way ANOVA, $F_{2,26} = 46$; $p < 0.001$) over the course of 4 dark phases and a significant increase of immobility time at dark phase 4 (Tukey post hoc analysis, $p = 0.0022$). Together, the results demonstrate a habituation effect to the novel environment and consistency between high locomotion and low immobility periods and vice versa.

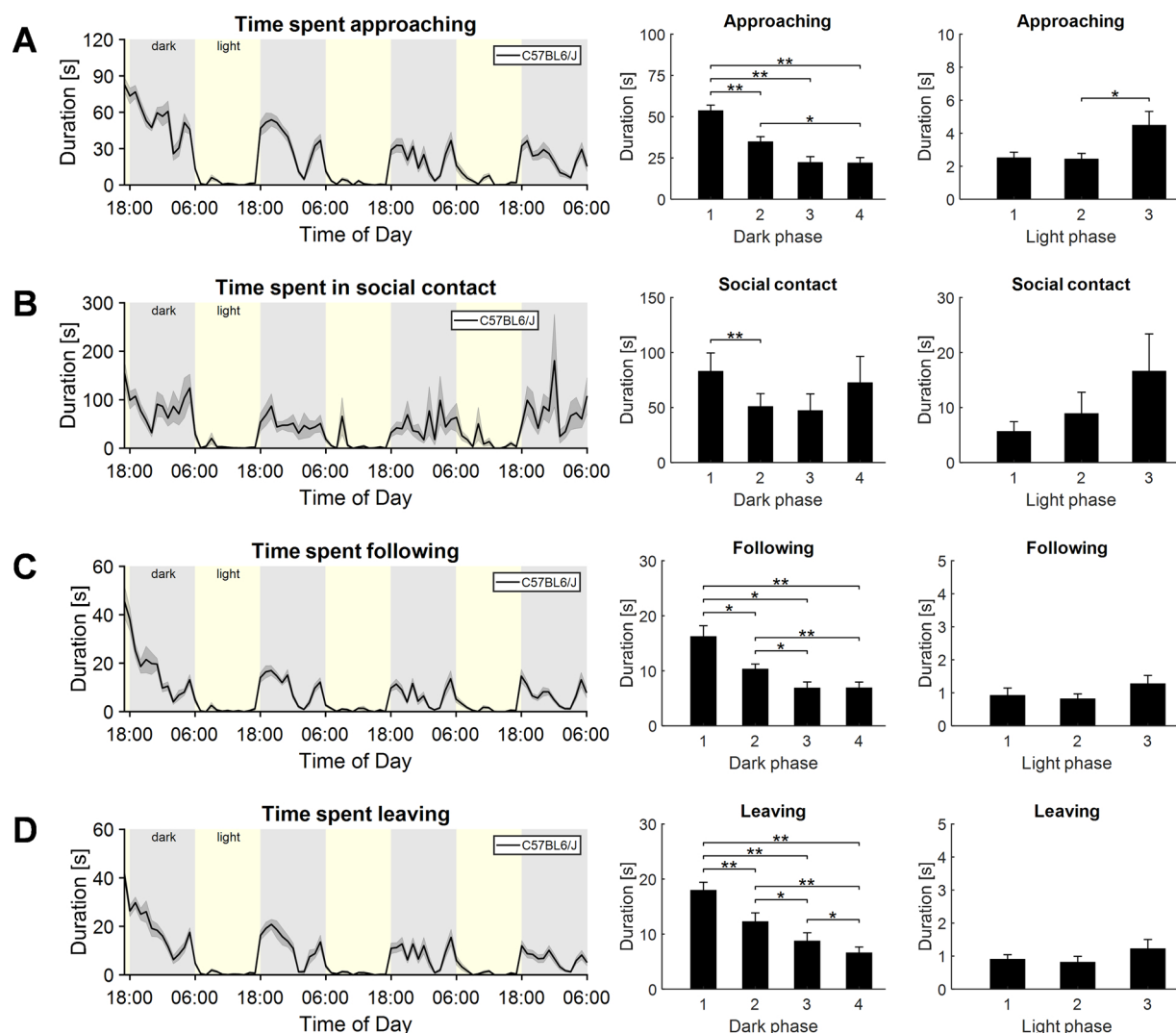


Fig. 7. Social behaviours in groups of C57BL/6 J mice. Duration (in seconds) of approach (A), social contact (B), follow (C) and leave (D) in groups of C57BL/6 J mice. Line graphs represent mean duration per 1 h over 84 h. Bar charts display mean duration per dark/light phase. Data are mean (\pm SEM) with $n = 16$ (with 4 mice per colony). * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA and Tukey's post hoc test.

3.3. Social behaviours

Fig. 7 displays duration of approach, contact, follow and leave in 4 colonies ($n = 16$ mice) of male C57BL/6 J mice over 84 consecutive hours. While approach (one-way ANOVA, $F_{2,23} = 24$; $p < 0.001$), follow (one-way ANOVA, $F_{1,18} = 13$; $p = 0.0014$) and leave (one-way ANOVA, $F_{2,25} = 34$; $p < 0.001$) behaviour significantly decreased over the course of four dark phases, social contact was not significantly different between dark phase 2 and 3 (Tukey post hoc analysis, $p = 0.5472$) and dark phase 3 and 4 (Tukey post hoc analysis, $p = 0.1968$). We calculated the number of social approaches of each mouse towards colony members during the dark phases (Fig. 8). Over the course of four dark phases, social approach pattern changed in all colonies. However, we observed an inhomogeneous behavioural pattern between colonies at dark phase 2 whereas during dark phase 4 all mice seem to approach each other equally.

4. Discussion

Common tracking systems are limited in detecting more than two subjects, depend on manual annotation of swapped identities, on the use of visible marking, and require permanent visibility of the subjects and/or are designed for bespoke behavioural setups.

We developed a RFID-supported video tracking tool that overcomes these limitations by (1) the ability to continuously detect four individual mice (larger groups are currently being tested), (2) automated correction of identity swaps, (3) using permanent and invisible RFID tagging, (4) identifying subjects outside the camera view (e.g. when entering nests) and (5) the possibility to create versatile environmental conditions in a flexibly modifiable behavioural apparatus.

The analysis software is commercially available under the trade name *RFID-Assisted SocialScan* (CleverSyS. Inc, USA). The software synchronously aligns RFID data and video frames allowing automated behavioural monitoring of freely moving mice. The originality of the system is to automatically correct identity swaps enabling continuous monitoring of individuals over long periods of time without intervention by the user. Colour marking is a widely used method to differentiate laboratory animals (Dahlborn et al., 2013). However, it is a topic of dispute. Colour marking can be achieved using pens, markers, ink, hair dyes, bleaches, hair removal and (rarely) picric acid (Anthony et al., 1959; Burn et al., 2008; Deacon, 2006; Kulkarni et al., 2011; Ohayon et al., 2013; Spink et al., 2001; Walker, 2012). Application of hair colour or bleaches, for instance, requires restraint or anaesthesia to apply the agent and to allow taking effect (Ohayon et al., 2013). Generally, colour marking is a temporal labelling method and requires re-application after a short period of time (Burn et al., 2008; Deacon,

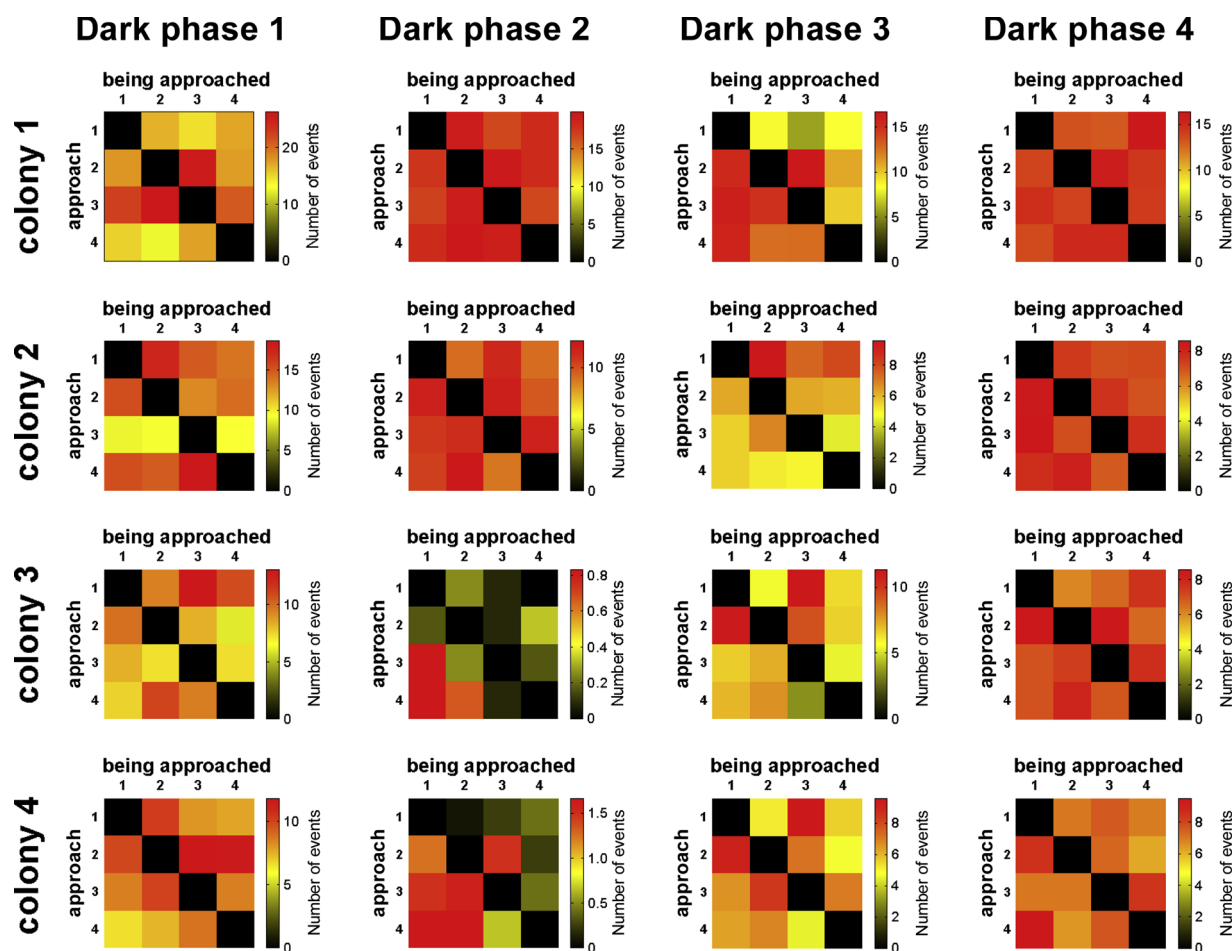


Fig. 8. Individual approach pattern in groups of C57BL/6J mice. Mean number (per hour) of social approach events during four consecutive dark phases. Numbers 1–4 represent individual mice approaching (rows) and being approached (columns). Scale was set automatically for every dark phase and colony. Thereby, black colour indicates no social approaches whereas red colour represents maximum number of approaches conducted during the dark phase.

2006). However, it is a matter of debate what (negative) effects on animal behaviour and health some of these marking methods might implicit. Tail marking with ink, for instance, was associated with higher chromodacryorrhea (red lacrimal secretion) responses in rats and simultaneously reduced anxiety in the elevated plus-maze (increased open arm entry) (Burn et al., 2008). Furthermore, little is known about how animals, in particular mice, perceive colour marking and how much this interferes with social behaviour. Lacey et al., for instance, reported that male mice marked with hair dye, were more likely to be subordinate (Lacey et al., 2007). For these reasons, RFID tagging provides multiple advantages: it is invisible, permanent and requires short exposure to anaesthesia (< 5 min, in our hands). RFID tracking allows detecting animals even when they are not seen by the camera. In our behavioural setup, for instance, mice are detected when entering nests attached to the arena. However, our social arena is designed in a flexible manner allowing attachment of other components resulting in variable behavioral setups for multiple behavioural applications. The ability to flexibly set behavioural definitions offers full control over the measurement scope and allows addressing multiple questions regarding social and non-social behaviours deriving from a single behavioural assay. This is not only compliant with the 3R principle (replace, refine, reduce) (Russell and Burch, 1959), it also saves resources and time to elaborately conduct multiple short-term experiments under artificial environmental conditions.

In the present study, we measured locomotor activity and social behaviours (approach, contact, follow and leave) over multiple days. We showed that locomotion and social behaviours decrease over days

and follow the circadian activity pattern of mice with peaks of low and high activity levels during the dark phase. The high levels of behavioural activity during the first day that decline during adaptation to the testing arena are consistent with earlier observations of individual home cage environment behaviour in C57BL/6J mice (de Visser et al., 2006; Kas et al., 2009). Furthermore, we observed dynamic changes between colony mates across dark phases and variability between colonies. However, further analysis is required to understand whether these dynamics changes result from individual variability in behavioural trajectories or whether they are influenced by the establishment of a social hierarchy (Freund et al., 2013; Uhrich, 1938).

Future work will be to determine reproducibility of our automated tracking system across laboratories using BTBR T + tf/J mice, a well characterized mouse model of autism with deficiencies in social behaviours (Peleh et al., manuscript in preparation).

5. Conclusion

We present here a novel automated tracking system for long-term behavioural monitoring of freely moving mice housed in a flexibly modifiable behavioural apparatus. We believe our system will be a valuable tool for a wide range of behavioural applications in basic research to study social behaviour and its dynamics and for translational research to validate clinically relevant biological substrate of e.g. social withdrawal for the development of new therapeutic concepts.

Declaration of competing interest

X. Bai is employed by CleverSys., Inc, which manufactures and commercializes the *RFID-Assisted SocialScan* for automated behavioural tracking. T. Peleh and B. Hengerer are employed by Boehringer Ingelheim Pharma GmbH & Co. KG. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Acknowledgements

The PRISM project (www.prism-project.eu) leading to this application has received funding from the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No 115916. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA. This publication reflects only the authors' views neither IMI JU nor EFPIA nor the European Commission are liable for any use that may be made of the information contained therein.

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